Determination of residue levels of clothianidin and its metabolites TZMU and TZNG in pollen harvested from maize plants grown in commercial practice from Poncho Pro® dressed seeds (nominally 1.25 mg clothianidin/seed) in the Upper Rhine Valley in Germany

Report:	Staedtler, T. (2009) Determination of the Residue Levels of Clothianidin and its Metabolites TZMU and TZNG in Pollen Harvested from Maize Plants Grown in Commercial Practice from Poncho Pro Dressed Seeds (Nominally 1.25 mg Clothianidin/Seed) in the Upper Rhine Valley in Germany: Final Report. Project Number: 071, R08188/2, EBTOL040. Unpublished study prepared by RIFCon GmbH. 140 p.
Document No.:	MRID 48298801
Guideline:	Non-guideline field residue study
Statements:	The study was conducted in compliance with Good Laboratory Practice – under German Chemical Law (Chemikaliengesetz), current OECD principles of Good Laboratory Practice, ENV/MC/Chem(98)17:Environmental health and Safety Publications; OECD guidance document ENV/JM/MONO (2002)9: Application of OECD principles of GLP to organization and management of multi-site studies; OECD Guidance document ENV/JM/MONO (99)22: Application of the GLP principles to field studies. These are generally compliant with FIFRA GLP standards under 40 CFR part 160.
Classification:	This study is **SUPPLEMENTAL.** It is uncertain if the pollen samples were representative of the area of the fields where the sampled bees were foraging. Further, location information for the bee hives is lacking with respect to potential foraging habitat. A phylogenetic analysis is needed to determine the source of the pollen that foraging bees are transporting back to the hive(s). Additionally, the study did not include information about the stability of clothianidin and its degradates during frozen storage, and transport.
PC Code:	044309

Primary Reviewer: Reuben Baris, Environmental Scientist
USEPA/OPP/EFED/ERB6

Signature: _____ **Date:** 10/02/2012

Final Reviewer: William P. Eckel, Senior Science Advisor
USEPA/OPP/EFED/ERB6

Signature: _____ **Date:** 10/2/12

Executive Summary

The objective of the study was to determine the levels of clothianidin residues and those of its metabolites thiazolylmethylurea (TZMU) and thiazolylnitroguianidine (TZNG) in corn pollen collected from plants grown from seeds dressed with clothianidin (Poncho Pro®; 1.25 mg a.i./seed), from pollen traps fitted to bee hives located next to selected sampling fields, from combs out of these hives, and in dead bee samples from traps fitted to these hives.

A total of 252 samples of pollen were taken from five corn fields planted with clothianidin treated corn seed[1], and an additional 193 samples from bee derived matrices (bee bread, pollen from pollen traps, and dead bees) were collected during the field sampling phase of the study (Table S1, pg. 8); bee-derived matrices were only collected from hives placed near three of the five fields (Bühl-Oberbruch, Kippenheim, Müllheim). Residues of clothianidin, TZNG and TZMU in corn pollen, bee bread and dead bee samples were extracted using an acetonitrile:water (4:1, v:v) mixture in an ultrasonic bath, "cleaned-up" using a Chemelut® column, dried by evaporation and taken up in toluene:ethyl acetate (85:15, v:v) and further "cleaned-up on a Silica Gel™ (SiOH) column. Residues were quantified by reversed-phase HPLC with Turbo-Ionspray MS/MS-detection. The Limit of Quantification (LOQ) for clothianidin, TZNG and TZMU was 1.0 ug/kg for all sample materials; the Limit of Detection (LOD) was 0.3 ug/kg for all samples.

The experimental (field) phase of the study was conducted over a period of 20 days (July 9 – 29, 2008). The analytical phase overlapped with the experimental phase beginning July 17th, and ending November 14th. The study authors did not provide detailed information about the storage of samples prior to analysis other than all samples were stored in a freezer soon after sampling until they were sent to the residue analysis laboratory of Bayer CropScience AG (pg. 24).

Corn pollen sample analysis resulted in 237 samples that contained clothianidin residues >LOQ (94% detection frequency, mean residue of 3.4 µg/kg; 90th %-tile residue of 5.2 µg/kg). The maximum detection in corn pollen was 10.4 µg/kg. Residue analysis of pollen from pollen traps placed at the entrance to bee hives resulted in 28 samples that contained clothianidin residues > LOQ (n=118, 24% detection frequency, mean residue of 1.1 µg/kg, 90th %-tile residue of 2.2 µg/kg). The maximum detection in pollen from pollen traps was 11.4 µg/kg. Dead bee residue analysis resulted in two samples >LOQ (1.2 and 1.1 µg/kg, n=39, mean residue of 0.5 µg/kg, 90th %-tile residue of 1.0 µg/kg). Both of these samples were obtained from hives that were designated to the same field region (Müllheim); it is unclear if the bees were from the same hive. Residue analysis of bee bread resulted in 7 samples >LOQ (n=36, mean residue of 1.0 µg/kg, 90th %-tile residue of 2.0 µg/kg). Results from the residue analyses are summarized in Table 1.

[1] **Bühl-Oberbruch, Schwanau, Kippenheim, Herbolzheim, Müllheim.**

Table 1. Mean residue concentration of clothianidin, TZNG and TZMU for five locations in the Upper Rhine Valley[†]

	Clothianidin (µg/kg)	TZNG (µg/kg)	TZMU (µg/kg)
Maize pollen*			
Bühl-Oberbruch	3.906	0.622	0.482
Schwanau **	2.854	0.622	0.300
Kippenheim **	3.358	0.524	0.454
Herbolzheim	3.942	0.664	0.370
Müllheim	2.942	0.440	0.384
Overall mean residue concentration (n=252)**	3.399	0.572	0.397
Pollen from pollen traps			
Bühl-Oberbruch	1.142	0.339	0.300
Schwanau	-	-	-
Kippenheim	1.605	0.479	0.318
Herbolzheim	-	-	-
Müllheim	0.730	0.475	0.300
Overall mean residue concentration (n=118)	1.137	0.435	0.306
Dead bees			
Bühl-Oberbruch	0.358	0.300	0.300
Schwanau	-	-	-
Kippenheim	0.475	0.300	0.300
Herbolzheim	-	-	-
Müllheim	0.507	0.507	0.300
Overall mean residue concentration (n=39)	0.451	0.379	0.300
Bee bread			
Bühl-Oberbruch	1.225	0.300	0.300
Schwanau	-	-	-
Kippenheim	1.333	0.475	0.300
Herbolzheim	-	-	-
Müllheim	0.550	0.442	0.300
Overall mean residue concentration (n=36)	1.036	0.406	0.300

[†] Obtained from Table 4, pg 29 of study report.
[*] sampled directly from maize plants
[**] The additional samples collected for data validation (n=2) could not be dedicated to one location named above as they were pooled samples from different locations (Schwanau and Kippenheim) but are included in the calculation of the overall mean concentration

I. Material and Methods

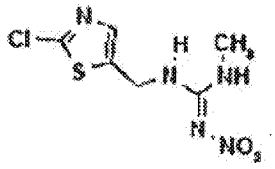
A. Materials

1. Test Material:

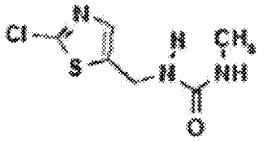
Product identification
Trade name: Poncho Pro®
Type of formulation: FS
Active substance content: 600 g a.s./L
Proposed use: Insecticide

Common name: Clothianidin
Development code: TI-435
Chemical name: (E)-1-(2-Chlor-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine
CAS registration no.: 210880-92-5

2. **Reference Compounds:** Listed below, extracted from the study on page 86-88 (attachment 1).

· Name of the Compound	Clothianidin (TI 435)
· Certificate of Analysis	AZ13320, dated 2006-04-18
· Mol-ID	114
· Structure	
· Chemical Name of the Compound	(E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine
· Product Code	AE 1283742 00 1B99 0001
· Empirical Formula	$C_6 H_8 Cl N_5 O_2 S$
· Molar Mass	249.68 g/mol
· Purity	99.4%
· Expiry Date	April 2009
· Batch No.	KTS10061-1-1

· Name of the Compound	TZNG
· Certificate of Analysis	AZ13254, dated 2006-02-27
· Structure	
· Chemical Name of the Compound	((1E)-1-amino-2-nitro-2-azavinyl)[(2-chloro(1,3-thiazol-5-yl))methyl]amine
· Product Code	AE 1303036 00 1B99 0001
· Empirical Formula	$C_5 H_6 Cl N_5 O_2 S$
· Molar Mass	235.65 g/mol
· Purity	98.6%
· Expiry Date	February 2009
· Batch No.	M24920

- Name of the Compound	TZMU
- Certificate of Analysis	AZ13775, dated 2006-10-30
- Structure	
- Chemical Name of the Compound	1-(2-chloro-thiazol-5-ylmethyl)-3-methyl-urea
- Empirical Formula	$C_6 H_8 Cl N_3 O S$
- Molar Mass	205.7 g/mol
- Product Code	AE 1303037-PU-01
- Purity	98.3%
- Expiry Date	October 2010
- Batch No.	M13993

B. Methods

1. Site Conditions

a. Study Site (extracted from pg. 20 of the study report):

The study was conducted at five locations in the Upper Rhine Valley (state of Baden-Wurttemberg, Germany, Figure 1 and Figure 2, pp. 21-22). Each of these five locations was in an area associated with one of the following municipalities:

- Buhl-Oberbruch (Appendix 1-1, Appendix 1- 2 and Appendix 1- 3)
- Schwanau (Appendix 2-1, Appendix 2- 2 and Appendix 2- 3)
- Kippenheim (Appendix 3- 1)
- Herbolzheim (Appendix 4-1, Appendix 4- 2 and Appendix 4- 3)
- Mullheim (Appendix 5- 1)

The locations of the various study plots were selected to ensure a representative distribution throughout the respective study field. A total of 5 pollen samples were collected from each study field (one sample from each plot within the field). At three of the five study locations (Buhl-Oberbruch, Kippenheim and Mullheim), bee hives were set up in order to conduct an independent bee monitoring study (non-GLP; Dr. Liebig, University of Hohenheim) in parallel to the current study. At these locations the study fields were partly chosen in the vicinity of the bee hives.

b. Applications

In each of these locations corn seeds commercially dressed with Poncho Pro® (a.i. clothianidin, nominally 1.25 mg a.i./seed) were sown in April/May 2008 (non-GLP, details see Appendix 1- 6, Appendix 2- 6, Appendix 3- 4, Appendix 4- 6 and Appendix 5- 4). At each location ten commercial maize fields (with maize plants grown from Poncho Pro® dressed seeds) were selected as study fields for pollen sampling (Appendix 6- 2). On each study field five individual

study plots were identified and pollen was collected from a number of individual plants within each plot (between 2 and 70 plants). Application rates (lbs a.i./A) were calculated based on the seeding rates for each field at each study site provided by the study authors in Appendices 1-5 of the study report. Table 2 summaries the calculated application rates for each study site.

Table 2. Summary of Calculated Application Rates for Clothianidin.

Site	Average Seeding rate from 10 fields (seeds/ha)	Seeds/acre	mg ai/seed	lbs a.i./A
Buhl-Oberbruch	80889	199796	1.25	0.55
Schwanau	59500	146965	1.25	0.40
Kippenheim	83800	206986	1.25	0.57
Herbolzheim	79700	196859	1.25	0.54
Mullheim	80780	199527	1.25	0.55
MEAN	**76934**			**0.52**

c. Weather (pg. 30 of the study report)

Weather data for the study sites during the field sampling phase were obtained from two different locations. Mean, minimum and maximum temperatures and precipitation for the northern part of the study site were provided by the Deutscher Wetterdienst (www.dwd.de) from their monitoring station at Karlsruhe. Similar weather data for the southern part of the study area were provided by the LTZ (Landwirtschaftliches Technologiezentrum Augustenberg), from their monitoring station at Mullheim.

The daily temperature in the northern part of the study area varied between 10.6°C and 34.2°C over the study period (09.07.2008 - 29.07.2008). Precipitation was recorded on ten days over this period with a total of 32.1 mm. On most of these days precipitation was < 5 mm; heavy rain occurred on the 11.07. (9.7 mm) and again on the 17.07. (8.2 mm) (Figure 3, pg 30 of the study report). For the southern part of the study area, temperatures range between 7.8°C and 30.7°C. Precipitation was recorded on six days during this period with a total of 48.0 mm. Rainfall > 5 mm occurred on the 11.08. (18.6 mm), the 13.07. (8.8 mm) and again on the 17.07. (14.2) mm. When compared to the long term weather data available for the northern region (1991-2007), minimum and maximum daily temperatures in the northern part of the study site were very similar to the long term average. Whilst the overall amount of precipitation was lower than normal for the study period, the number of days on which precipitation occurred was slightly more than the long term average. For the southern part of the study, compared to the long term weather data available for July in the region (1961-1990), mean daily temperatures in the southern part of the study site were slightly higher than the long term average. Conversely, precipitation for the month of July was similar to the long term average.

2. Residue Sampling:

a. Pollen from Corn (pp. 22-23 of study report)

Ten fields were selected for each region (Bühl-Oberbruch, Schwanau, Kippenheim, Herbolzheim, Müllheim). Within each field, pollen samples were collected from a number of corn plants located within five equally distributed plots[2] (the reviewer notes that in the respective appendices the sample plots were not identified on the field maps with respect to bee hive location). For each sampling plot panicles were cut from a number of corn plants (between 2 and 70 plants) and immediately placed in a paper bag to provide a composite sample from the respective plot. A minimum of 2 g pollen were collected from each plot. Only the minimum number of plants necessary was sampled to obtain a 2 g sample amount. The number of plants on each plot required to produce this amount of sample varied and was dependent on both the degree to which pollen was being shed and the local weather conditions.

Once collected, individual panicles from each plot were transferred to a second paper bag and shaken for a few seconds in order to break their anthers and induce the shedding of pollen (Appendix 6- 4, pg. 69). This process was repeated with all the panicles from an individual plot to provide a composite sample for the respective plot. Plant debris and other unwanted material was subsequently removed from each sample using a graduated series of fine sieves (2 mm, 1 mm, 0.5mm, 0.315 mm) and featherweight forceps (Appendix 6- 5, Appendix 6- 6, pp. 69-70). The cleaned samples were then transferred to a sealable container (PE, capacity of 50 ml). All sample containers were marked with a sticker detailing the study number, individual sample ID, date and sampling location. Further information (weight, number of plants used for sampling, details according sampling storage) was recorded on an appending data sheet, marked with the sample's individual number. Some samples were stored indoors for drying overnight, in which case shaking, cleaning, and extracting of pollen was completed the following morning. Four samples were placed in a drying cabinet due to wet weather conditions during sampling. The reviewer notes that these samples were not identified in the results, nor were supplemental data provided regarding the stability of clothianidin for these storage conditions.

All samples were stored in a freezer at the accommodation directly after processing until sent for residue analysis to the laboratory of Bayer CropScience AG. Freezer temperatures were recorded daily throughout the whole storage period using a minimum/maximum thermometer. During shipment samples were stored in dry ice and temperature during transport was recorded by means of a data logger placed in the cooling container. Supplemental data was not provided regarding the stability of clothianidin for these storage conditions.

b. Bee Derived Matrices (pp. 23-24 of study report)

Samples were also taken from the bee hives set up next to the study fields. The hives were set up in three of the five study locations (Buhl-Oberbruch, Kippenheim and Miillheim) near the study fields as part of a bee health monitoring study undertaken in parallel to the current study (Dr. Liebig, University of Hohenheim; non-GLP). At each location twelve bee hives were set up prior to the onset of the blooming of the maize plants (Appendix 6- 7, pg. 71).

[2] One plot was consistent with one sample.

In order to determine clothianidin residues in corn pollen collected by the bees, pollen traps (devices to remove the pollen pellets from the incoming bees' legs and a container to collect these pellets) were installed in front of 9 bee hives (colonies 1 - 9, respectively; Appendix 6- 8, pg 71). Pollen deposited in these traps was recovered on selected days during the pollen sampling activities in the maize fields and afterwards (Appendix 6- 9, pg 72). Pollen/bee bread was sampled directly from honey combs (once from each hive, Appendix 6- 10, pg. 72) using a knife to cut the respective samples out of the comb. Additionally, traps to collect dead bees (removed from the hives by other bees) were installed in front of three bee hives at each location (colonies 10 - 12, respectively; Appendix 6-11, pg 73) and dead bees were recovered for residue analysis. Each sample was in either a sealed container (PE, capacity 50 ml) or a plastic bag. All sample containers were marked with a sticker detailing the study number, individual sample ID, date and sampling location. Further information (bee hive identity number, sample type, weight) was recorded on an appending data sheet, marked with the sample's individual number.

It is important to note that pollen from the pollen traps were not phylogenetically analyzed for source analysis (*i.e.*, it was not confirmed that the pollen captured in the traps was from the treated fields).

All samples were stored in a freezer at the accommodation as soon as possible until sent for residue analysis to the laboratory of Bayer CropScience AG. Freezer temperatures were recorded daily throughout the storage period using minimum/maximum thermometer. During shipment samples were stored on dry ice and temperature during transport was recorded by means of a data logger placed in the cooling container. The review notes that storage stability data for clothianidin was not provided in the study report for the longest storage period, therefore it is uncertain if residues of clothianidin were degraded under these storage conditions.

3. Analytical Procedures

a. Residue Analysis (pg. 24 of study report)

For all samples collected during the Field Phase, clothianidin residues and those of its relevant metabolites, TZMU and TZNG, were analyzed by the laboratory of Bayer CropScience AG according to the analytical method No. 00554/M001 (Modification M001 of the residue analytical method 00554 for the determination of residues of TI-435, TZNG and TZMU metabolite in nectar (honey) and pollen by HPLC with electrospray MS/MS detection (Schoening 2001)).

b. Residue Analysis and Extraction Methods

Residues in the maize pollen, bee bread and dead bee samples were extracted using a mixture of acetonitrile/water (4/1; v/v) in an ultrasonic bath. The extracts were cleaned-up using a ChemElut® column. After evaporation to dryness the samples were taken up in toluene/ethyl acetate (85/15; v/v) and further cleaned-up on a Silica Gel™ (SiOH) column. The residues were quantified by reversed-phase HPLC with Turbo-Ionspray MS/MSdetection. The evaluation was

performed using internal stable labeled standards. Reference standards, and chemical structures are listed on pages 86-88 of the study report.

Detection limits, Limit of Detection (LOD) and Limit of Quantification (LOQ), were determined to be 0.3 µg/kg and 1.0 µg/kg, respectively.

II. Results and Discussion:

Fifty pollen samples were obtained from each of the five study locations to provide a total of 250 samples. Additionally 2 samples for validation of the methodology were collected. In each case it was intended to recover a minimum of 2 g pollen for each sample. However, in 22 cases this target was not achieved because the maize panicles collected on the plots provided insufficient amounts of pollen. Table 1 on page 25 of the study report provides detailed dates on the sampling timing and duration.

A total of 193 samples of bee-derived matrices were obtained from the three sampling locations at which hives were set up. The exact dates of sampling and the respective sample types are given in Table 2 (pg 25) of the study report. Samples of pollen from pollen traps and dead bees were recovered over several days whereas bee bread was collected once from each hive. Additional, detailed information on the bee hives is given in Appendix 6-1 (pg 66).

A. Findings

Table 2 summarizes the number of analyzed samples and categorizes the number of detections in each category as <LOD, <LOQ but > LOD, between the LOQ and < 3 µg/kg, 3-10 µg/kg, > 10 µg/kg, respectively for each sampling matrix. Mean clothianidin residue in corn pollen ranged from 2.85 to 3.94 µg/kg, with an overall mean of 3.4 µg/kg (n = 252). Mean residues in the pollen sampled in the pollen traps[3] ranged from 0.73 to 1.61 µg/kg from hives in the three regions sampled, overall mean was 1.14 µg/kg (n = 118). Mean residues of clothianidin in bee bread ranged from 0.55 to 1.33 µg/kg, overall mean of 1.04 µg/kg (n = 36). Mean residues in dead bees in hives from three regions ranged from 0.36 to 0.51 µg/kg, overall mean of 0.45 µg/kg (n = 39). Calculation of mean residue concentrations values <LOQ were assumed to be 0.001 mg/kg, and values <LOD to be 0.0003 mg/kg. These were the assumptions provided in the study report and provide a conservative estimate of mean residue concentrations in the sampled matrices. Table 1 summarizes the mean concentrations of clothianidin and its two degradates TZNG and TZMU in the sampled matrices. Analytical results (residue concentrations) of all samples are detailed in the Analytical Phase Report and corresponding Amendments (see Attachment 1-4).

Table 3. Number of analyzed samples with their concentrations of clothianidin, TZNG and TZMU

[3] It is important to note that a phylogenetic analysis was not completed on the pollen extracted from pollen traps to determine the source of the pollen.

	Clothianidin	TZNG	TZMU
Maize pollen* (n=252)			
<LOD	11	154	217
<LOQ	4	97	35
<3 µg/kg	79	1	-
3-10 µg/kg	157	-	-
>10 µg/kg	1 (10.4 µg/kg)	-	-
Pollen from pollen traps			
<LOD	38	97	117
<LOQ	52	18	1
<3 µg/kg	23	3	-
3-10 µg/kg	4	-	-
>10 µg/kg	1 (11.4 µg/kg)	-	-
Dead bees			
<LOD	31	35	39
<LOQ	6	2	-
<3 µg/kg	2	2	-
3-10 µg/kg	-	-	-
>10 µg/kg	-	-	-
Bee bread			
<LOD	11	31	36
<LOQ	18	4	-
<3 µg/kg	5	1	-
3-10 µg/kg	2	-	-
>10 µg/kg	-	-	-

* sampled directly from maize plants

III. Study Deficiencies and Reviewer's Comments

Deficiencies:

1. The study did not provide information about the stability of clothianidin and its degradates during frozen storage. There were as few as eight days between the initiation of the field phase and the initiation of the analytical phase, and as much as 121 days between the completion of the field phase and the completion of the analytical phase. Data is needed on the frozen storage conditions, and the stability of clothianidin and its degradates for the longest storage period. The study authors discuss the daily recording of freezer temperatures, however this data was not provided in the study.
2. The study lacks information on the location of the bee hives with respect to the treated fields. Location information is needed to determine if there are additional forage habitats for the bees other than corn.
3. The source of the pollen in the traps is not known, therefore bee exposure to clothianidin treated corn is uncertain. A phylogenetic analysis is needed to determine the source of the pollen that foraging bees are transporting back to the hive(s). This analysis would decrease the uncertainty surrounding the exposure route of bees to treated seed. It is uncertain if the bees are being exposure to the active ingredient through the pollen. These data show that the compound(s) are trans-located within the plant and are detected in the pollen.

4. It is uncertain if the pollen samples were representative of the area of the fields where the sampled bees were foraging. The location of the sampling (number of plants from each area)

Comments:

1. Mean residues were calculated assuming detections < LOD equaled the LOD, and detections > LOD, but < LOQ equaled the LOQ.